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TITLE: Novel Role of Merlin Tumor Suppressor in Autophagy and its Implication in Treating NF2-Associated Tumors

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14. ABSTRACT

We have recently demonstrated that Merlin regulates the induction of autophagy, a cellular catabolic pathway implicated in the maintenance of cellular homeostasis. Deregulated autophagy is linked to a number of human disease conditions, including tumorigenesis. Loss of Merlin leads to attenuated autophagy and consequent elevation of metabolic stress, a condition known to accelerate tumor formation in vivo. This metabolic stress can be suppressed by an autophagy inducer rapamycin. During the first year of this grant award, we demonstrated that Merlin interacts with several autophagy-related proteins (i.e., LC3, Ulk1, and dynein) using the heterologous expression system. In addition, cell biological analyses showed that autophagy induction is attenuated in cells that express Merlin/K79E, a point mutation found in NF2 patients. In the second year, we have extended our analysis to confirm that endogenous Merlin interacts with LC3, Ulk1, and dynein, in mouse embryonic fibroblasts (MEFs). Furthermore, LC3-Merlin-dynein complex formation was attenuated in Ulk1-KO MEFs, demonstrating a critical role of Ulk1 in the assembly of these proteins upon autophagy induction. We have also established the 3D culture system to evaluate the extent of metabolic stress caused by loss of Merlin function. Using this system, we will further characterize the effects of autophagy inducers in modulating metabolic stress and tumorigenesis in Merlin-deficient cells.

15. SUBJECT TERMS

Autophagy induction, metabolic stress, Atg1, rapamycin

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Introduction:

Our preliminary study demonstrated that Merlin promotes autophagy, a cellular clearance system responsible for degrading old proteins or damaged organelles within cells, and thus help mitigate the risk of tumor formation. Moreover, the cellular stress caused by loss of Merlin function was effectively suppressed by rapamycin, an autophagy-inducing compound. Therefore, we hypothesize that Merlin normally suppresses tumorigenesis in part by activating autophagy, and that this new role of Merlin in autophagy could be a target for therapeutic intervention against NF2-associated tumors. To test this hypothesis, we have evaluated the interaction of Merlin with autophagy-related proteins (i.e., LC3, dynein, Ulk1/Atg1). We will also analyze how Merlin mutations found in NF2 patients may affect the autophagy-inducing activity of Merlin. These analyses are expected to provide new insight into the role of Merlin in autophagy and to contribute to the development of new therapeutic means against NF2.

Body:

Interaction studies of Merlin and autophagy-related protein (Aim 1)

The following items (1a. through 1e.) were accomplished during the 1st year of the grant cycle, as already reported in the first annual report (April 2012).

1a. Preparation of expression constructs
1b. Transfection and immunoprecipitation experiments
1c. Data analysis for 1a and 1b
1d. Setting up mouse crosses to prepare MEFs
1e. Preparation of MEFs

During the second year of the grant cycle, we have extended the work to MEFs cells, which were prepared in Aim.1e during the 1st year, in order to firmly establish the interaction of Merlin with autophagy-related components.

1f. Transfection of MEFs and interaction assays

MEFs were prepared from pups (+/*GFP-LC3*$^+$, embryonic day 14.5) born from ♂(+/+) x ♀(+/*GFP-LC3*$^+$) mouse mating pairs, and GFP-LC3-positive MEFs were transduced by lentiviruses encoding Merlin-knockdown short-hairpin RNA (shRNA) and LC3-Merlin-DIC interaction assay was done by immunoprecipitation (**Fig. 1**). Similar to the results shown in the 1st year annual report using exogenously expressed proteins in HEK293T heterologous expression system, GFP-LC3 were found to interact with Merlin, and pulled down dynein intermediate chain (DIC) from control MEFs. In addition, this LC3-Merlin-DIC association was upregulated upon autophagy induction by nutrient starvation. However, when Merlin was knocked down, interaction between GFP-LC3 and DIC was abolished, demonstrating Merlin-mediated, autophagy-dependent complex formation among LC3, Merlin, and DIC.

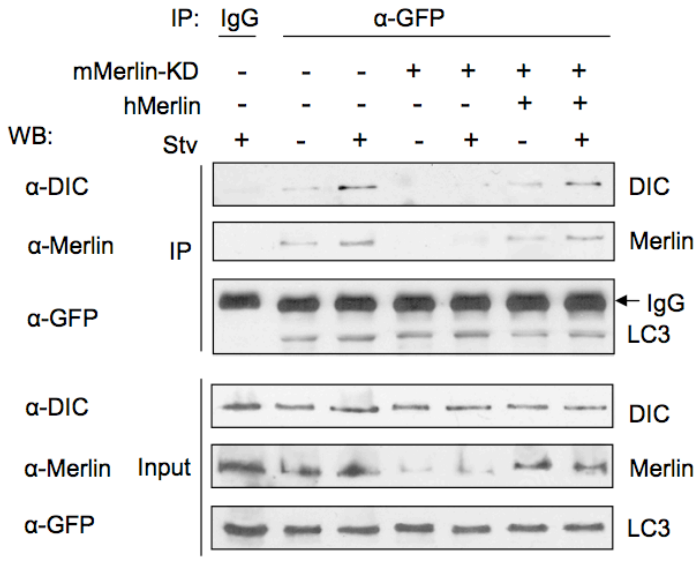


Fig.1 Merlin assembles LC3 and Dynein upon autophagy induction

Wild-type MEFs that stably express GFP-LC3 were transduced by lentiviruses encoding Merlin-knockdown shRNA, and the resulting cell lysates were analyzed by immunoprecipitation (IP) with anti-GFP (α-GFP) followed by Western blots (WB), using the indicated antibodies. DIC (dynein intermediate chain). Human Merlin transgene (hMerlin) was expressed, as necessary, via infection of retroviruses expressing hMerlin. Autophagy was induced by nutrient starvation (Stv) in Earle's balanced salt solution for 15 min.

In order to investigate biochemical roles of Ulk1/Unc51.1/Atg1 in the LC3-Merlin interaction during autophagy induction, we prepared *Ulk1*-KO MEFs from pups (*Ulk1*$^{-/-}$;+/*GFP-LC3*$^{+}$, embryonic day 14.5) born from ♂(*Ulk1*$^{+/-}$;+/+) x ♀(*Ulk1*$^{+/-}$;+/*GFP-LC3*$^{+}$) mouse mating pairs, and GFP-LC3-positive *Ulk1*-KO MEFs were nutrient starved to induced autophagy. The resulting cell lysates were analyzed by IP followed by WB (**Fig.2**). Although Merlin-LC3 interaction was upregulated upon autophagy induction in control MEFs as expected from the result in Fig.1, the degree of Merlin-LC3 interaction was unchanged before and after autophagy induction in Ulk1-KO MEFs, indicating that Ulk1 is responsible for the induction of LC3-Merlin interaction during autophagy progression.

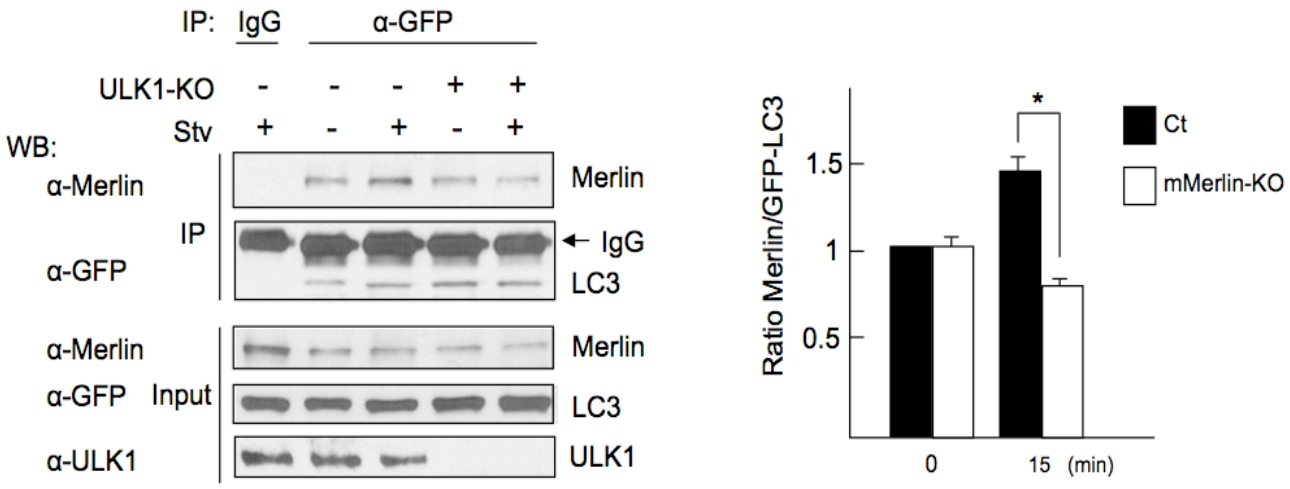
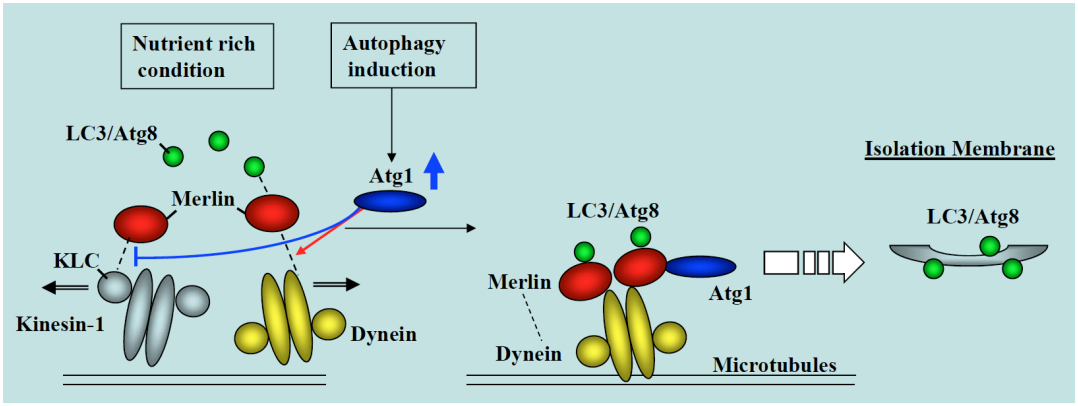


Fig.2 Merlin-LC3 interaction is upregulated during autophagy induction and the interaction depends on Ulk1/Atg1 activity

Wild-type or Ulk1-KO MEFs that stably express GFP-LC3 were nutrient-starved (Stv) for 15min to induce autophagy and the resulting cell lysates were immunoprecipitated (IP) with anti-GFP (α-GFP) and analyzed by Western blots (WB) using the indicated antibodies. The assays were done in triplicate, and the average ratio of Merlin/LC3 was plotted in the graph.

1g. Data analysis for 1f

Based on the IP experiments done using the heterologous expression system (1a. – 1e.) (reported in the 1st year annual report), as well as additional IP experiments using MEFs shown above (1f.), we hypothesize that Merlin serves as an adaptor protein that links LC3 autophagy protein and Dynein motor, which helps deliver LC3 toward the pre-autophagosomal structure (isolation membrane). Upon autophagy induction, or when Ulk1/Atg1 kinase activity is upregulated, Merlin forms a complex with LC3 and Dynein. As shown in the 1st year annual report, Ulk1/Atg1 kinase activity downregulates Merlin-KLC interaction, and upregulates Merlin-Dynein interaction, facilitating the delivery of LC3 more toward the retrograde direction.



Evaluation of autophagy activity of mutant Merlin (Aim 2)

2a. Preparation of expression constructs

We have completed the construction of all the mammalian expression constructs necessary to carry out this experiment, which include myc-tagged Merlin/WT, K79E, E270G mutants.

2b. Transfection and immunocytochemistry experiments

In order to evaluate the effect of Merlin mutants on autophagy induction, we followed a standard lipofection procedure to transfect these constructs into wild-type MEFs that stably express GFP-LC3 transgene. Formation of GFP-LC3-positive puncta was observed every 20 min after nutrient starvation, using fluorescence microscopy, and the average numbers of GFP-LC3 puncta were scored by randomly choosing ~30 cells per each condition. At every time point, cells were fixed with 4% paraformaldehyde in PBS, and the transfected cells were identified by immunostaining cells with anti-myc antibody to visualize the expression of exogenously transfected Merlin transgene. MEFs that expressed Merlin^{K79E} showed significantly fewer numbers of GFP-LC3-positive puncta, as compared with control MEFs or MEFs expressing Merlin^{E270G}, suggesting that K79E mutation inhibits Merlin-mediated autophagy induction in a dominant-negative manner (**Fig.3**).

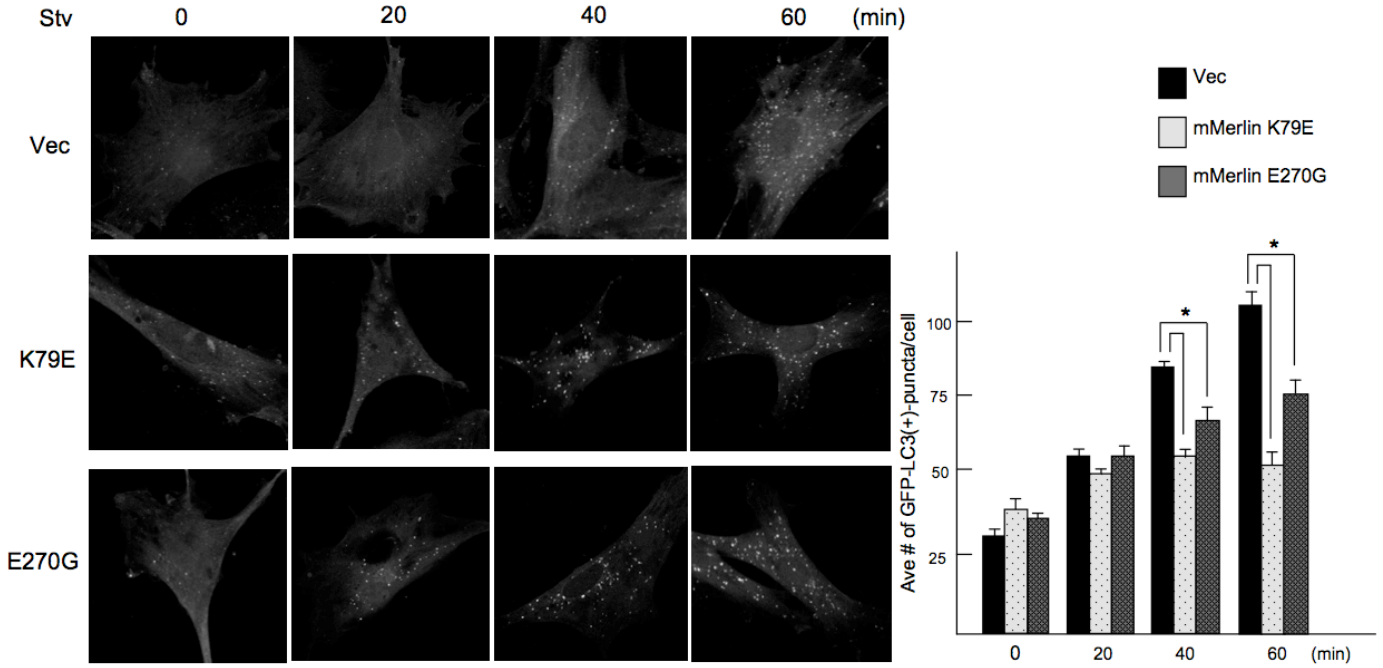


Fig.3 MEFs that stably express GFP-LC3 were transfected with Merlin (WT, K79E, E270G) and the autophagosome formation was observed after nutrient starvation (Stv) in Earle's balanced salt solution up to 1 h. The numbers of GFP-LC3-positive puncta per cell were scored.

2c. Data analysis for 2a and 2b

We confirmed that Merlin^{K79E} negatively regulated autophagy induction upon nutrient starvation. In a separate biochemical experiment, we confirmed that Merlin^{K79E} was unable to bind LC3, whereas Merlin^{E270G} maintained the ability to interact with LC3 (data not shown). On the other hand, Merlin^{K79E}, when expressed in MEFs, suppressed the cellular proliferative activity as effective as Merlin^{WT}, whereas Merlin^{E270G} lost the ability of growth suppression as measured by BrdU incorporation assay (data not shown). Taken together, the role of Merlin in autophagy seems independent of its growth suppressive activity.

2d. Setting up mouse crosses to prepare Ulk1/Atg1$^{-/-}$ MEFs

This was accomplished during the 2nd year of the grant award, as initially planned in the Statement of Work. In brief, mouse mating pairs (♂(*Ulk1*$^{+/-}$;+/+) x ♀(*Ulk1*$^{+/-}$;+/*GFP-LC3*$^{+}$)) were set up and *Ulk1*$^{-/-}$;+/*GFP-LC3*$^{+}$ embryos (E14.5) were chosen to prepare GFP-LC3-positive *Ulk1*-KO MEFs.

2e. Preparation of MEFs

This was accomplished as described above (2d), and already used in IP-WB experiments (1f).

Evaluation of autophagy inhibitors in 3D culture system (Aim 3)

3a. Preparation of MCF10A cells

Three dimensional (3D) culture system using MCF10A cells was established during the 2nd year of the grant award. In brief, MCF10A cells were first maintained in monolayer cultures in growth media (DMEM/F12 plus 5% horse serum, 10mg/ml insulin, 20ng/ml EGF, 100ng/ml choleratoxin, 0.5ug/ml hydrocortisone, 100U/ml penicillin, and 100mg/ml streptomycin, then the 3D cultures of MCF10A were prepared on Matrigel in growth media lacking epidermal growth factor (EGF) and with reduced horse serum (2%). To induce autophagy, amino acid-free starvation media (Earle's balanced salt solution), or complete media with rapamycin (100nM) were used. Autophagy inhibitors used were: 3-methyladenine (10mM) and bafilomycin A1 (50nM).

3b. Initiate 3D cultures

MCF10A 3D cultures were prepared by seeding $1x10^4$ MCF10A cells from the monolayer culture onto a Matrigel-coated culture chamber slide. Under this condition, single cell of MCF10A would develop into a sphere-like structure called acini within 10 to 14 days in vitro. Genetic manipulation, such as Merlin knockdown or Ulk1/Atg1 expression, can be accomplished by infecting MCF10A cells with a lentivirus encoding Merlin-knockdown shRNA or with a retrovirus expressing Ulk1, respectively, at the beginning of the culture when cells are at the single cell stage, and the transduced cells were selected by puromycin selection (2ug/ml) for 3 days.

3c. Drug treatment of MCF10A 3D cultures

MCF10A cell 3D cultures on Matrigel were treated with a series of autophagy inducers or inhibitors for the last 6 days of the culture period, to evaluated cellular response (*i.e.*, the extent of metabolic stress as estimated by DNA damage response (phospho-histone 2A.Ser-139 [pH2A.X.] or by hypoxia marker [hpi]), cell growth (Ki-67 proliferation marker immuno-staining), or a cell-shape marker (β-catenin antibody)) (**Fig.4**). These data demonstrated that loss of Merlin resulted in a higher level of cellular proliferation (higher Ki-67), as well as higher levels of metabolic stress, as evidenced by higher hpi staining and pH2A.X staining (**Fig.5**), as compared with control knockdown cells (Ct-KD). Rapamycin (Rapa) treatment was able to significantly suppress cellular proliferation and the degree of metabolic stress.

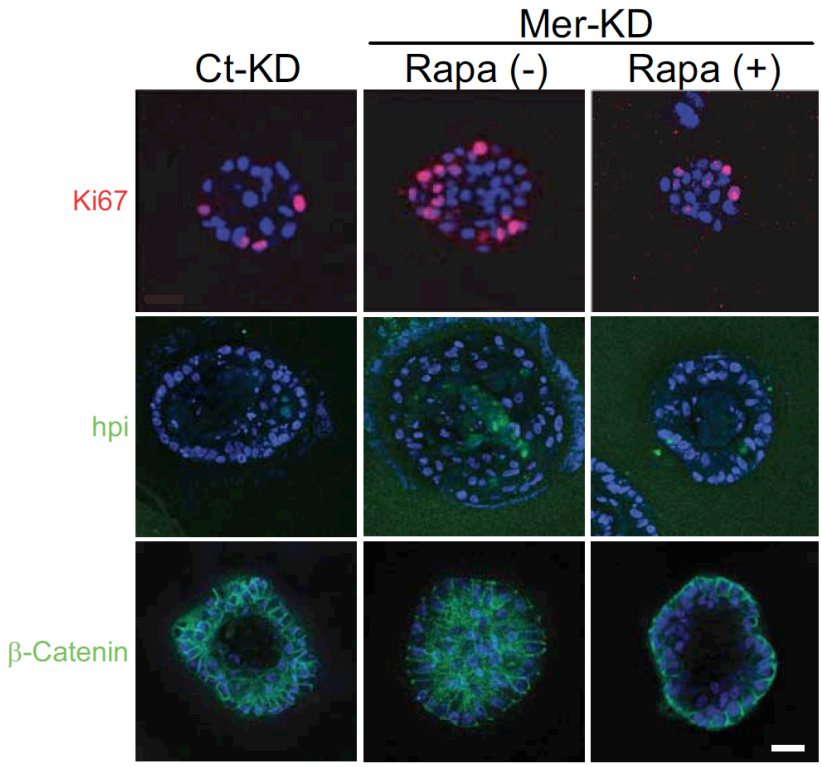


Fig.4 MCF10A spheres were stained with anti-Ki67 (red) at 12DIV, anti-Hypoxyprobe-1 (green) at 14DIV, or anti-β-catenin antibodies (green) at 10DIV, as well as with DAPI (blue). Each sphere was observed by confocal microscopy and the optical section through the mid-plane of the sphere is shown. Scale Bar: 40um

MCF10A spheres were infected with the lentivirus encoding the control (Ct-KD) or Merlin knockdown (Mer-KD) shRNA. The cultures were treated with rapamycin for the last 6 days in culture, fixed and stained with the indicated antibodies.

To determine the extent to which autophagy manipulation could influence metabolic stress seen in Merlin-KD 3D cultures, autophagy was either upregulated by ULK1/wt, or downregulated by ULK1/dn expression, or pharmacologically inhibited by bafilomycin A1 or 3-methyladenine (3MA). Enhancing autophagy reduced the level of metabolic stress, whereas inhibition of autophagy enhanced metabolic stress in 3D cultures (Fig.5). These results are consistent with the role of autophagy in tumor suppression, and suggest that activation of autophagy pathway could serve as a therapeutic strategy against tumors in which Merlin-mediated autophagic activity is lost or attenuated.

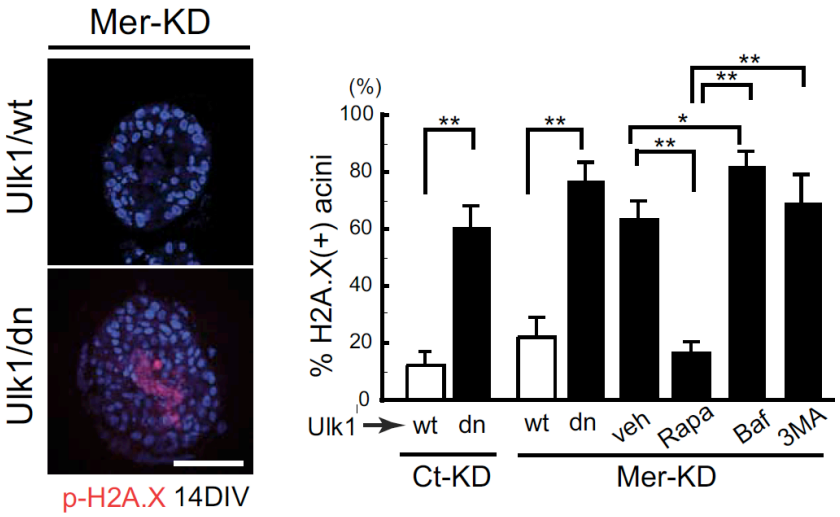


Fig.5 MCF10A 3D cultures were treated with autophagy inducers/inhibitors as indicated, and the extent of metabolic stress (DNA damage response) was evaluated using anti-p-H2A.X antibody.

MCF10A spheres were doubly infected with the control (Ct-KD) or Merlin shRNA lentivirus and with the retrovirus expressing either wild-type ULK1 (ULK1/wt) or dominant-negative ULK1 (ULK1/dn), and stained at 14DIV with anti-pH2A.X (red) and DAPI (blue). Scale bar: 40um

Graph: MCF10A spheres infected as in (E) were treated with rapamycin (Rapa), bafilomycin A1 (Baf), 3-methyladenine (3MA), or vehicle (Veh), and the rate of spheres with pH2A.X-positive cells (%) were scored (mean ± SEM). Statistical significance (*; $p<0.05$, **; $p<0.005$; Student's *t*-test)

Key Research Accomplishments:

- Merlin promotes the accumulation of the LC3 autophagy-related protein on the autophagic membrane precursor called isolation membrane, by serving as a linker between LC3 and dynein motors.

- NF2-associated mutation Merlin^{K79E} specifically inhibits autophagy induction without affecting its role as a growth suppressor, while the additional mutation, Merlin^{E270G}, affects the growth suppressive function of Merlin protein and did not significantly affect autophagy.

Reportable Outcomes:

- PI presented this work at the oral session at Childrens' Tumor Foundation (CTF) annual Meeting held at New Orleans (June 2012).

- Dr. Akiko Sumitomo was hired (June 2012).

- This work was presented by Ms. Yuki Hirota at the annual poster session held at City of Hope (Nov. 2012).

- PI presented this work at the 6th International Symposium on Autophagy held at Okinawa (Nov. 2012).

- The research paper describing part of the above results was submitted to a journal in Jan, 2013, and has been under revision to be considered for publication as of Aug. 2013.

Conclusion:

By extending the analyses performed during the first year of the grant period, we successfully confirmed the novel role of Merlin in promoting autophagy. We showed that Merlin is a part of multi-protein complex that serves as a scaffolding machinery to promote autophagic membrane assembly. At least one of the NF2-associated mutation (Merlin^{K79E}) was found inhibitory to autophagy induction, while an additional mutation Merlin^{E270G} did not significantly affect autophagy. This result demonstrates the dual role of Merlin tumor suppressor in growth suppression and autophagy induction, both of which are relevant to NF2-related tumorigenesis. Further analyses are needed to delineate the role of Merlin in autophagy.

References:

None

Appendices:

None

Supporting Data:

Five figures and one schematic diagram are included in the body of this report.